Identification and characterization of microRNAs in vascular smooth muscle cells from patients with abdominal aortic aneurysms

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Background: **The role of microRNAs (miRs) in the development of various cardiovascular diseases was recently highlighted in several studies. However, the biological role of miRs in the pathogenesis of abdominal aortic aneurysms (AAAs) is still not well defined. The present study aims to identify and characterize miR expression in the aortic explant cultures of AAA patients.**

Methods: **A new microarray platform (miChip) using locked nucleic acid-modified capture probes with increased sensitivity and specificity was employed to detect the miR expression profile in human vascular smooth muscle cell cultures from AAA surgical samples. The array data were further validated by real-time quantitative reverse transcription polymerase chain reaction (qPCR) experiments with additional AAA and control aorta samples. Potential target genes of the differentially expressed miRs were predicted by bioinformatics analysis. Some of the results were confirmed by enzyme immunosorbent assay and Western blot analysis.**

Results: **Total RNA extracted from the explant of six AAAs and six normal aortas exhibited notably different miR profiles. A twofold difference of miR-516a-5p and miR-1260 was expressed in AAAs compared with normal aortic cultures ($P < .05$). The expression of miR-516a-5p was more than three times higher in AAAs, and miR-1260 expression was almost four times lower, as validated by qPCR. Additional qPCR performing on an extra 10 AAAs and 10 control aortas showed similar results. The expression of three predicted targets—secretory interleukin-3, vascular endothelial growth factor A, and collagen type 1, alpha 1 proteins—was significantly elevated in aneurysmal cultures compared with normal aortic cultures ($P < .05$).**

Conclusions: **This miR microarray study displayed an altered expression of miR-516a-5p and miR-1260 in AAAs compared with control aortas. Functional annotations of the two miRs via bioinformatics approaches revealed that both are highly involved in some predefined mechanisms of AAA formation. Three of their target genes were also upregulated in AAAs. The results may be critical to elucidate the functional role of miRs in diseased aorta. (J Vasc Surg 2014;59:202-9.)**

Clinical Relevance: **MicroRNAs are newly found pathologic factors for many cardiovascular diseases. However, its involvement in abdominal aortic aneurysms (AAAs) is still a mystery. The novel findings of two differential miRs associated with their target protein expression in aneurysmal tissues may be important in delineating partly the pathophysiology of AAAs. The manipulating of the miRs in AAAs may then become a new therapeutic concept in the future.**

Abdominal aortic aneurysm (AAA) is a common dilating disorder of the aorta and a major cause of death due to rupture.[1] The majority of previous studies on human tissue have focused on some predefined pathways such as chronic inflammatory condition with a proteolytic imbalance, but the complex regulation of gene expression and translation involved in driving aneurysmal formation and growth remain unresolved. A group of microRNAs (miRs) have recently emerged as new study target for precise gene regulation.[2] They are highly conserved, 21 to 23 nucleotides in length, and typically encoded within introns, which regulate gene expression in animals at the post-translational level. By annealing to the 3' untranslated region (UTR) in the target messenger RNAs, the miRs mostly mediate translational repression or degradation of the mRNAs, resulting in downregulation of the protein level. In addition, miRs are also able to stimulate gene expression.[3] More than 1000 miRs have been identified in the human genome (miRBase v. 16.0). In fact, bioinformatic analysis predicts that each miR regulates hundreds of targets, which suggests that miRs may play a role in almost every biological pathway, including developmental timing and patterning, differentiation, proliferation morphogenesis, and apoptosis.[3] In conclusion, miR represents a new layer of precise regulation for sequence-specific gene expression. The importance of the miR regulatory pathways is also underscored by an impressive list of diseases found to be associated with abnormal miR expression. Evidence is accumulating that many age-related diseases are associated with a decreased control of cell signaling, DNA repair, oxidative stress responses, and apoptosis.[4-7] With significant progress in miR microarray technology, numerous sets of miRs have been found to be highly or specifically expressed in aortic tissues with

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particular functions. To date, research on the biological importance of miRs in human AAAs is still very scarce, with, for example, only 14 studies identified when using *AAA* and *microRNA* as keywords to search PubMed.

The present study aimed to analyze the miR expression patterns of human vascular smooth muscle cells (VSMCs) obtained from AAA explant cultures and to compare them with control aortas using the highly sensitive and specific miRCURY microarray platform. Altered expression of individual miR was further validated by quantitative real-time polymerase chain reaction (qPCR). If the biological importance of miRs in the pathogenesis of AAAs can be confirmed, these results may lead to new therapeutic strategies for AAA in the future.

METHODS

Human abdominal aorta tissue collection. Medial sections of aneurysm wall samples, which were dissected of luminal thrombus and adventitia, were acquired from AAA patients who underwent open surgical aneurysmal repair in a local hospital. Control aortic tissues from the corresponding location were obtained at liver transplantation procurement. All liver donors had no known cardiovascular diseases and connective tissue disorders. Aneurysmal patients with Marfan syndrome or other connective tissue disorders were excluded from this study. Each collected specimen was thoroughly washed with Dulbecco's modified Eagle's medium (DMEM) solution and cultured in DMEM (Invitrogen, Carlsbad, Calif). The remaining specimens were kept in a −80°C freezer until protein analysis. All experiments were performed with the approval from the local institution's ethics committee. Informed consent was obtained from AAA patients and organ donors' relatives.

Aortic organ explant cultures. Both aneurysmal and normal aortic walls were washed free of blood and transported to a sterile tissue culture hood in DMEM (Invitrogen). The aortic tissues were divided into 1- to 2-mm^2 segments of medial wall and were placed into six separate wells of tissue culture plates, supplemented with 3 mL of DMEM containing 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 4 to 7 days, before the cultured cells reached confluence, the cells were harvested for gene and protein study. Conditioned media were collected and stored at −20°C for secretory cytokine detection using enzyme-linked immunosorbent assay (ELISA). The homogeneity of VSMCs expressed in the explant cultures was confirmed by histochemical staining with anti-α-actin antibody (Cat. No. M0851; Dako, Glostrup, Denmark).

Total RNA isolation. Total RNAs were isolated using Trizol reagent (Invitrogen) and further purified using an RNeasy mini kit (Qiagen, Valencia, Calif). RNA quality control was performed using Bioanalyzer 2100 in Genome Research Center, The University of Hong Kong. Purified total RNAs (with good quality) were sent to Exiqon (Vedbaek, Denmark) for miR expression analysis. MiR microarray analyses including labeling, hybridization, scanning, and normalization were carried out by Exiqon.

miR profiling using miRCURY LNA microarray. In brief, 500 ng of total RNA from each specimen was labelled with an Hy3™ and Hy5™ fluorescent label, respectively, using the miRCURY locked nucleic acid (LNA) array power labelling kit (Exiqon) following the instructions from the manufacturer. Similarly, the Hy3™-labelled samples and an Hy5™-labelled reference RNA sample were mixed pair-wise and hybridized to the miRCURY™ LNA array version fifth generation (Exiqon), which contains capture probes targeting all human miRs in the miRBASE v.16.0 at the Sanger Institute (Cambridge, UK). The hybridization was performed according to the miRCURY LNA array manual using a Tecan HS4800 hybridization station (Tecan, Grödig, Austria). Three independent hybridizations for each sample were performed on chips with each miR spotted in quadruplicate. Labeling efficiency was evaluated by analyzing the signals from control spike-in capture probes. This set of LNA-modified oligonucleotides was designed to have uniform melting temperatures of 72°C against their complementary targets. After hybridization, the microarray slides were scanned and stored in an ozone-free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc, Santa Clara, Calif), and image analysis was carried out using the ImaGene 9.0 software (BioDiscovery, Inc, Hawthorne, Calif). The quantified signals were background corrected (Normexp with offset value 10) and normalized using the global LOWESS (LOcally WEighted Scatterplot Smoothing) regression algorithm. Fold changes in miR expression levels from independent arrays were calculated and presented as means. Putative targets of the miRs were predicted using the online software MiRANDA.

qPCR for altered miR expression. The expression levels of altered miRs and one reference gene were further validated by qPCR using the miRCURY LNA microRNA PCR system and SYBR Green master mix, following the manufacturer's instructions (Exiqon). qPCR was performed using an Applied Biosystems 7900 Sequence Detection system (Applied Biosystems Inc, Foster City, Calif). Potential gene targets were retrieved from the miR target prediction database, miRANDA. Three predicted targets that were thought to be involved in inflammation and vascular remodeling were selected for further expression analysis.

The expression levels of any differentially expressed miRs detected in the Exiqon microarray platform were first validated in the corresponding samples using individual real-time qPCR assays. Then, the qPCR assays were further performed on explant cultures from an extra set of AAA patients and control aortas for better understanding of the characteristics of the differentially expressed miRs.

Quantification of proinflammatory mediators using ELISA. The secretory levels of cytokine interleukin (IL)-3 and vascular endothelial growth factor A (VEGFA), which

were the two proinflammatory mediator targets of the upregulated miR516a-5p, were detected in the culture media by the Quantikine kit (R&D Systems, Abingdon, UK).

VSMCs collection and lysis for specific protein-expression analysis. VSMCs were harvested and mixed with lysis buffer (Cell Signaling Technology, Danvers, Mass) containing protease inhibitor (Roche, Basel, Switzerland) for protein-based assays. After centrifugation at 4°C for 15 minutes at 13,000 rpm, the supernatants were collected and stored at −80°C until use.

Western blot analysis of COL1A1. The protein expression of collagen type 1, alpha 1 (COL1A1), one of the target genes for the downregulated miR1260, was analyzed in the VSMCs of aneurysmal explant cultures and compared with that of normal aorta.

The protein concentration of each specimen was measured based on the Bradford method, using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, Calif) with bovine serum albumin as the standard. After denaturing the protein with loading buffer (pH 6.8 24 mM Tris-HCl, 684 mM glycerol, 14 mM SDS, 142 mM beta-mercaptoethanol, 0.3 mM bromophenol blue), each sample (50 μg) was resolved on 12% SDS-polyacrylamide gel electrophoresis gel (Bio-Rad Laboratories) at room temperature and then transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories) at 4°C. Subsequently, each sample was blocked in 10% TBS−0.01% Tween 20 diluting nonfatty milk (Bio-Rad Laboratories) for 2 hours at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies against COL1A1 (catalogue no. AF6220; R&D Systems, Abingdon, UK, 140 kDa) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH; catalogue no. 2118, 1:10000 dilution; Cell Signaling Technology, 37 kDa) as positive control. After membrane washing using TBST solution, HRP-conjugated secondary antibody (Dako) was added, and the membranes were then incubated for 1 hour at room temperature. After washing, signals were visualized by luminol reagents (GE Bio-Rad Laboratories) and the densitometry of each exposing blotting was analyzed by ImageJ 1.44 software (National Institutes of Health, Bethesda, Md). The relative expression of the studied collagen protein was calculated by the detected signal divided by the internal positive control (GAPDH) expression signal in each sample.

Statistical analysis. Results are expressed as mean ± standard deviation (SD). Comparisons between two groups were made by Student *t*-test. *P* values of <.05 or less were considered significant.

RESULTS

Expression profiles of miRs in AAAs and normal aortas with altered genes identified. Six male AAA patients and six sex-matched organ donors with normal aortas were recruited for this study. The demographic data of the patients are summarized in Table I. The hybridization signals of miR expression levels ranged from several thousand to a few hundred units. Several expression patterns of miRs were found to be significantly different between these two groups. Only those genes that showed good reproducibility and reliability and with alteration tendency ($P < .05$ and fold change >2) were selected as differentially expressed genes. A short summary list of differentially expressed miRs is shown in Table II. Out of all the miRs expressed on the array, only two miRs with significantly altered expression were identified. Compared with normal aortic explants, an elevated miR-516a expression and a reduced miR-1260 expression were identified in the AAA explants, with the highest fold change and statistical difference.

Table I. Characteristics of AAAs and control groups

	AAA (n = 6)	*Control (n = 6)*
Age, years	69.5 ± 8.2	42.2 ± 9.2
Males/females	6/0	6/0
Aneurysm diameter, cm	7.56 ± 1.17	-
Hypertension	1 (16.7%)	-
Smoking	5 (83.3%)	-

AAA, Abdominal aortic aneurysm.

Confirmation of array data using qPCR. qPCR on the two identified miRs was first performed in the corresponding samples to verify the array results. The relative fold changes in the expression of the miRs as detected by qPCR assays validated the microarray results. The increase of the expression of miR-516a-5p was more than threefold and miR-1260 had an almost fourfold decrease, as validated by qPCR.

Additional qPCR analyses were performed on 10 AAA samples (six elective AAAs and four ruptured AAAs) and 10 control aortas. All samples were male and comparable in age (AAA patients' mean age = 62 ± 12 years old, five smokers; control organ donors' mean age = 59.6 ± 6 years old, eight nonsmokers, smoking histories of two organ donors were missing). miR-1250a-5p was upregulated fourfold in the AAA tissues compared with the control samples, and miR-1260 was downregulated fourfold in AAAs compared with control samples. The extra qPCR results were consistent with the Exiqon results.

Among all of the AAA samples, no significant differences in miR516a-5p or miR-1260 expression could be detected between the aneurysm explant cultures from elective or emergency open operation. No significant differences in the expression of these two specific miRs could be detected in any of the control aortic samples. This showed that the expression of miR516a-5p was not related to the smoking habits of the AAA patients. Collectively, all qPCR results (16 AAA samples and 16 control aortic samples) confirmed that the two differentially expressed miRs were not age- or smoking-dependent and were consistently expressed in the 16 AAA tissue samples or the 16 control aortas.

Targets of miRs and functional annotations by bioinformatics analysis. To further investigate the biological significance of the expressed miRs, functional annotations of the two miRs were analyzed by miRANDA

Table II. A summary of the miR profiling of AAA explants (n = 6) vs control aortic explants (n = 6) using miRCURY LNA-based microarray platform (Exiqon)

Probe ID	*Sample ID Group Annotation*	P *value*	*Average*		*SD*		*dLMR*	*SD*	*Fold change*
			A	*C*	*A*	*C*	*A-C*	*A-C*	*A vs control*
42550	hsa-miR-516a-5p	.01	0.15	−0.81	0.32	0.34	0.96	0.47	1.947
145975	hsa-miR-1260	.04	−0.93	0.13	0.63	1.11	−1.06	1.28	0.48
46755	hsa-miRPlus-E1026	.04	−0.45	0.08	0.46	0.35	−0.54	0.58	0.689
46498	hsa-miRPlus-F1099	.05	−0.37	0.01	0.08	0.36	−0.38	0.37	0.77
46624	hsa-miR-1236	.06	0.02	0.42	0.21	0.47	−0.4	0.51	0.759
145838	hsa-miR-125b-1	.06	−0.48	−1	0.49	0.24	0.53	0.55	1.441
46786	hsa-miRPlus-F1193	.06	0.16	−0.22	0.19	0.28	0.38	0.33	1.298
146196	hsa-miR-711	.07	−0.46	−0.95	0.32	0.26	0.49	0.41	1.403
11175	hsa-miR-525-5p	.07	0.36	−0.22	0.39	0.29	0.58	0.49	1.493
42661	hsa-miR-492	.07	−0.28	−0.68	0.28	0.17	0.39	0.33	1.311
46473	hsa-miRPlus-E1112	.1	0.21	−0.12	0.24	0.22	0.33	0.33	1.257
46256	hsa-miRPlus-E1238	.11	−0.33	−1.09	0.54	0.58	0.76	0.79	1.691
17354	hsa-miR-637	.11	−0.8	0.19	0.79	0.59	−1	0.99	0.501
45891	hsa-miRPlus-E1285	.12	−0.14	−0.75	0.48	0.41	0.61	0.63	1.529
146072	hsa-miR-1469	.14	−0.41	0.12	0.42	0.46	−0.53	0.62	0.691
46885	hsa-miRPlus-E1225	.15	0.33	−0.54	0.81	0.52	0.87	0.97	1.825

AAA, Abdominal aortic aneurysm; *dLMR,* absolute Δ LogMedianRatios; *miR,* microRNAs; *SD,* standard deviation.
Hsa-miR-516a-5p and mi-1260 showed the highest fold change ($P < .05$).

bioinformatics. The results indicated that they are highly involved in aortic stiffness, arterial wall changes, atherosclerosis, inflammation, hypertension, cytokines, and chemokine-dependent cell signaling. All these functions are predicted pathways for AAA formation (Tables III and IV).

Secretory level of IL-3 and VEGFA. IL-3 and VEGFA, were detectable in the culture media of both types of tissue cultures. The secretory levels were significantly increased in the aneurysmal explant cultures compared with those of normal aortas ($P < .05$) (Table V).

COL1A1 in VSMCs. COL1A1 protein expression, which is one of the predicted miR-1260 targets, was increased in the VSMCs of the aneurysm aortic explant cultures compared with those in the normal aortas ($P < .05$) (Fig).

DISCUSSION

Unlike conventional mRNA expression profiling, accurate miR profiling is technically challenging due to the small size of mature miRs and the sequence similarity between miR family members. In the present study, a distinct miR expression profile in the diseased human VSMCs was generated using the new miR microarray technology with high sensitivity and specificity.

The existing literature is very limited, and only 14 published studies were found on PubMed using the search terms of *microRNA* and *aneurysm*. In the extant research, miR-143 and -145 were found to positively regulate vascular smooth-muscle specific gene expression, differentiation, actin cytoskeleton, and contractile function.[8,9] miR27b and miR-130a were found to be proangiogenic,[10,11] and miR-221/222 were found to be regulators for VSMC proliferation and neointimal hyperplasia.[12] The study by Milewicz et al also found that decreasing levels of miR-29b in the aortic wall can attenuate aortic aneurysm progression in two different mouse models.[13] These studies highlighted the relevance of miRs in aortic disease. In addition, some studies indicated that miRs have distinct expression profiles and play crucial roles in various vascular pathologic processes such as aging,[14] remodeling, atherosclerosis, and inflammation.[15,16] To the best of our

Table III. The most aortic-related genes associated with the identified hsa-miR-516a-5p

Broad phenotypes	*Target gene*
(ANCA)-associated vasculitis	TNF
Abdominal aortic aneurysm	MTHFR
Hypertension	MTHFR
Arterial and venous thrombosis	MTHFR
Arthritis	TNF
Arthritis	IL-3
Arthritis, juvenile	TNF
Arthritis, psoriatic	NFKB1
Arthritis, psoriatic	TNF
Atherosclerosis, coronary	VEGFA
Atherosclerosis, coronary	PPP1R3A
Atherosclerosis, coronary	GPX1
Atherosclerosis, coronary	PTGS1
Atherosclerosis, coronary	TNF
Atherosclerosis, coronary	MTHFR
Atherosclerosis, coronary	CXCL12
Atherosclerosis, generalized	GPX1
Blood pressure	FURIN
Blood pressure, arterial	GNAS

CXCL 12, Chemokine (C-X-C motif) ligand 12; *FURIN,* furin; *GNAS,* stimulatory G-protein alpha subunit; *GPX1,* glutathione peroxidase 1; *IL-3,* interleukin-3; *MTHFR,* methylenetetrahydrofolate reductase; *NFKB1,* nuclear factor NF-kappa-B p105 subunit 1; *PPP1R3A,* protein phosphatase 1 regulatory subunit 3A; *PTGS1,* prostaglandin-endoperoxide synthase 1; *TNF,* tumor necrosis factor; *VEGFA,* vascular endothelial growth factor A. Source: miRANDA bioinformatics analysis, total 756 entries. The secretory protein levels encoded by the underlined genes — IL-3 and VEGFA — were analyzed.

Table IV. The most aortic-related genes associated with the identified hsa-miR-1260

Broad phenotypes	*Target gene*
Angina	GP1BA
Aortic stiffness	CYP11B2
Aortic stiffness	COL1A1
Arterial stiffness blood pressure, arterial	ECE2
Arterial wall changes	CYP11B2
Arthritis	TAP2
Atherosclerosis, carotid	AHSG
Atherosclerosis, coronary	LMNA
Atherosclerosis, coronary	PECAM1
Atherosclerosis, coronary	CYP11B2
Atherosclerosis, coronary lipoprotein	HAP1
Atherosclerosis, coronary myocardial infarct	GP1BA
Atherosclerosis, diabetes	PTPN1
Atherosclerosis, generalized	GP1BA

AHSG, Alpha-2-HS-glycoprotein; *COL1A1,* collagen, type I, alpha 1; *CYP11B2,* cytochrome P450, family 11, subfamily B, polypeptide 2; *ECE2,* endothelin converting enzyme 2; *GP1BA,* glycoprotein Ib (platelet), alpha polypeptide; *HAP1,* Huntingtin-associated protein 1; *LMNA,* lamin A/C; *PECAM1,* platelet endothelial cell adhesion molecule 1; *PTPN1,* protein tyrosine phosphatase, non-receptor type 1; *TAP2,* protein antigen peptide transporter 2.
Source: miRANDA bioinformatics analysis, total 774 entries. The expression of specific protein COL1A1 encoded by the underlined gene was analyzed.

Table V. The secretory levels of IL-3 and VEGFA in the culture media of control aortas explant cultures and AAA explant cultures

	IL-3	*VEGFA*
Control aortas	60 ± 15	125 ± 52
AAA specimens	350 ± 60[a]	452 ± 80[a]

AAA, Abdominal aortic aneurysm; *IL,* interleukin; *VEGFA,* vascular endothelial growth factor A.
[a]$P < .05$.

knowledge, the present study provides the first experimental results with functional annotations of the involvement of miRs in the explant cultures of clinical AAA samples. The available literature includes three recent miR studies on aneurysm walls. However, the specimens used were either full-thickness aneurysmal wall that are heterogeneous in terms of the cellular composition[17] or taken from animal models.[18,19] Pahl et al revealed that three miRs (miR-21, -146a, 181a) were upregulated in AAA surgical samples, while five miRs (miR-30c-2, -133a, -133b, -204, -331-3p) were significantly downregulated.[17] In the study by Liu et al, 10 mouse miRs (miR-19a, -19b, 34b, -92a, -132, -142-3p, -149a, -188, -221, -222) were upregulated and five (148b-39, 152, -181a, -301a, -497) were downregulated.[18] Another animal model found that AAA development was accompanied by decreased aortic expression of miR-29b, along with increased expression of known miR-29b targets, COL1A1 (which aligns with our results), COL3A1, COL5A1, and Eln.[19] In addition, the Maegdefessel research group tried to manipulate miR-29b and study the specific effect on murine abdominal aortic aneurysm development.[19] Interestingly, there were no common miRs found among the previous human or experimental AAA studies and also our explant cultures study. Milewicz collectively conferred the important role of miR-29 in aortic remodeling that probably involved in aneurysm formation.[13] However, miR-29 was not differentially expressed in our samples.

Specifically, the present miR expression platform profiles of all aortic samples were based on miRBASE 16 scanning for 1205 potential human miR target sequences. Another newly released human AAA study employed Affymetrix GeneChip miRNA 1.0 Array (Santa Clara, Calif), which contained 847 miR probes. Thus, the discrepancy of our results may be due to the different miR sources and also different miR expression platforms used in our study.

Identical results were found between intact and ruptured AAAs. Thus, these two specific miRs may not be markers for rupture predication. Collectively, all qPCR results (16 AAA samples and 16 control aortic samples) confirmed that the two differentially expressed miRs were not age or smoking dependent and were consistently expressed in AAA tissue or control aortas.

The putative targets of the two differentially expressed miRs were annotated by miRANDA bioinformatics. The numerous target genes predicted are mainly involved in aortic stiffness, arterial wall changes, atherosclerosis, inflammation, hypertension, cytokines, and chemokine-dependent cell signaling. These pathways are all involved in AAA formation.[20]

To understand the functional significance of the two identified miRs, validation of some interested predicted targets was performed. The expression of two proinflammatory mediators, namely IL-3 and VEGFA—which were the predicted targets of the upregulated miR516p-5a—and COL1A1—which was the predicted target of the downregulated miR1260—were examined accordingly. These three predicted targets were upregulated in the AAA explants compared with that in the control aortic specimens. The detectable targets in the culture cells may partially confirm the potential involvement of the two identified miRs in AAA pathogenesis.

First, the secretion of proinflammatory mediator IL-3, which is one of the predicted targets of the upregulated miR-516a-5p, was enhanced in the AAA explants. To date, IL-3's involvement in aneurysmal formation remains unknown, although many other cytokines, including interleukins IL-1, -2, -6, and -8, are thought to be important mediators for aneurysmal growth.[21] Interestingly, a research group found that IL-3 is frequently elevated in patients suffering from coronary arterial disease. Multivariate analysis showed that IL-3 concentration was the only independent parameter predicting symptomatic restenosis in patients undergoing percutaneous coronary intervention.[22] Previous studies also suggested that IL-3 plays a role in the pathophysiology of atherosclerosis. In human umbilical vein endothelial cells, IL-3 can stimulate the expression of P-selectin, a proatherogenic adhesion molecule that mediates rolling, adhesion, and transmigration

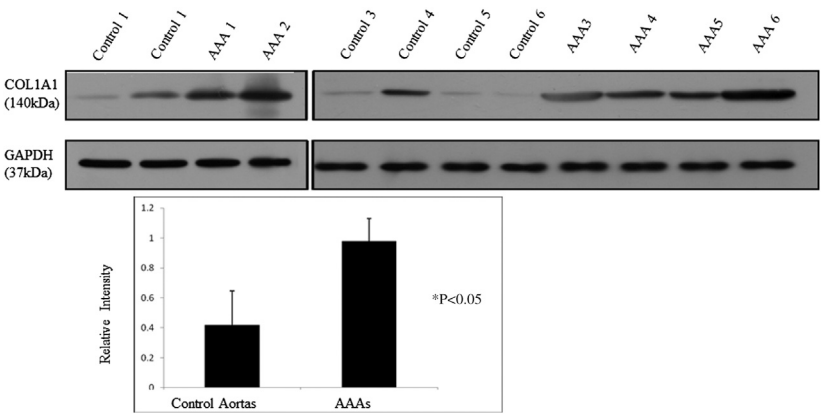

Fig. Upper panel, Representative Western blot results of collagen type 1, alpha 1 (*COL1A1*) protein expression in control aortas (*control 1-6*) and AAA specimens (*AAA 1-6*). COL1A1 protein was detectable in all control aortas and AAA specimens, in which the expression is significantly increased in AAAs compared with control aortas. **Lower panel,** Relative intensities of COL1A1 protein in control aortas and AAAs. COL1A1 protein levels were significantly upregulated compared with control aortic tissues ($P < .05$). *GAPDH,* Glyceraldehyde 3-phosphate dehydrogenase.

of leukocytes and monocytes.[23,24] Human IL-3 was also found to stimulate endothelial cell motility and promote in vivo new vessel formation.[25] Taken together, there is accumulating evidence suggesting that IL-3 is a relevant factor in the progression of the atherosclerosis that may be involved in AAA formation.

Second, the secretory levels of VEGFA, another predicted target of the miR-516-5p, were more increased in the AAA explant cultures than in the control aortas. Indeed, VEGFA is a member of the platelet-derived growth factor (PDGF)/vascular endothelial growth factor (VEGF) family. This protein acts specifically on endothelial cells, leading to such effects as increasing vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis. Alternatively spliced transcript variants, namely VEGFA, -B, and -C, have been characterized.[26] Recently, a Japanese research group showed an increased VEGFA levels in human AAAs and experimental mice AAAs.[27] Another recent study found a highly significant correlation between VEGFA and metalloproteinase (MMP)-2 in the progression of gastric cancer.[28] In addition, VEGF and MMP-9 might act synergistically to positively regulate angiogenesis.[29] MMP-2 and MMP-9 are the main metalloproteinases to degrade matrix protein and play a critical role in atherosclerosis and the pathogenesis of aneurysms.[20] Thus, the expression of these two downstream targets of the differentially expressed miR-516p further confirms the importance of miR516-5p and is particularly essential to the understanding of the cellular regulatory networks of matrix-degraded aneurysms.

Third, COL1A1 protein expression was found to be upregulated in aneurysms and consistent with the results of the study of Maefdefessel et al.[19] Indeed, type I collagen is the predominant fiber in the aortic wall that provides tissues with tensile strength and is believed to impart arterial stiffness. Wang et al hypothesized that high collagen content resulted in decreased arterial distensibility and increased susceptibility to aortic dissection.[30] To achieve the highly variable levels of type I collagen in different tissues during development, growth, aging, and tissue repair, the genes encoding the constituent α1 and α2 chains of type I collagen, COL1A1 and COL1A2 are likely to be under complex transcriptional and post-transcriptional control.[31] These elements were also found to mediate the complex effects of cytokines.[32]

Taken together, the two proinflammatory mediators may participate in the inflammatory and atherosclerotic processes in AAA formation, and the increased COL1A1 may be somehow mediated by cytokines, which is important for matrix remodeling in the aorta that ultimately led to aneurysm formation. Any correlation between the COL1A1 and cytokines definitely needs to be further investigated. However, miRs mostly mediate translational repression or degradation of the mRNAs, resulting in downregulation of the protein level and also the ability to stimulate gene expression. Therefore, the expression of these two downstream targets of the differentially expressed miR-516p may be negatively regulated by the intermediate cellular regulatory networks of the matrix-degraded aneurysm, but the underlying mechanism for the increased expression of the three downstream target genes remains unresolved.

Most recent miR studies focus on miR-29 in AAAs.[14,19,33] Those studies postulate that miR-29 is involved in aneurysm formation by post-transcriptionally repressing the expression of extracellular matrix proteins. Indeed, miR-29 targets several extracellular matrix proteins, which are known to maintain the integrity of the vascular wall.[14] MiR-29 was additionally shown to induce apoptosis in cancer cells by targeting myeloid cell leukemia sequence 1, an antiapoptotic B-cell lymphoma 2 family member.[6] Smooth muscle cell apoptosis is considered to favor aneurysm formation,[1] and this mechanism may contribute to

miR-29-mediated destabilization of the vascular wall. Thus, therapeutic inhibition of miR-29 using anti-miRs is suggested to be a novel therapeutic approach to interfere with vascular aging by augmenting matrix synthesis. Although the precise function of the presently detected miRs remains to be determined, our results may supplement the study of miR expression in AAAs.

One limitation of our study is that only samples from late stages of the human aneurysmal disease can be obtained from surgical intervention for this differential microRNA study. Most miRs are studied in experimental aneurysm models, which may account for the different miR expressions in different studies. However, our results may further consolidate the importance of miRs in the AAA pathogenesis.

In conclusion, increased miR-516-5p expression and decreased miR-1260 expression with the corresponding target expressions were observed in human AAA explant cultures compared with control aortas. Given the emerging role of miRs in physiological events and diseases, these two miRs may be crucial in AAA pathogenesis. The administration of anti- and overexpression of the two miRs in vivo using a lentiviral vector is currently under investigation in our laboratory to delineate the importance of the detected miRs and the involved pathways in the pathogenesis of AAAs. The present investigation can support the therapeutic manipulation of miRs and their target genes for limiting AAA disease progression.

AUTHOR CONTRIBUTIONS

Conception and design: BC
Analysis and interpretation: BC
Data collection: BC
Writing the article: BC
Critical revision of the article: BC, SC
Final approval of the article: SC
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Overall responsibility: BC

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